Results of MCD practical MDL1-3 – Enzyme kinetics

This practical class was composed of a "wet" part of around one hour which was dedicated to acquiring the data concerning NADH generation (measured by the spectrophotometer as an increase in A_{340}) and a "dry" part, where the resulting numbers were crunched in order to generate something meaningful from the absorbance measurements, via the plotting of two graphs.

A minority of students had problems with the pipettes, mostly with volume settings (P1000 for 0.2-1.0ml and P200 for 0.05-0.2ml) and in fact some made the error of using only approximately 10% of the enzyme/NAD+ volumes suggested. This was not a totally wasted exercise as it ably demonstrated that the reaction does not proceed at a useful rate without the enzyme and co-factor. Also, one or two couples used the wrong wavelength for NADH measurement. This should have been 340 nm as this is the optimal wavelength for measuring NADH absorption (see the graph on page 59 in your course guide). NAD^+ and the MDH enzyme should also have been kept on ice in the polystyrene buckets until their addition to the cuvette. A few students made up cuvettes in advance leaving the NAD^+ for prolonged periods at room temperature which rapidly lost its activity.

Below is a brief outline of the procedure used to analyse the raw data.

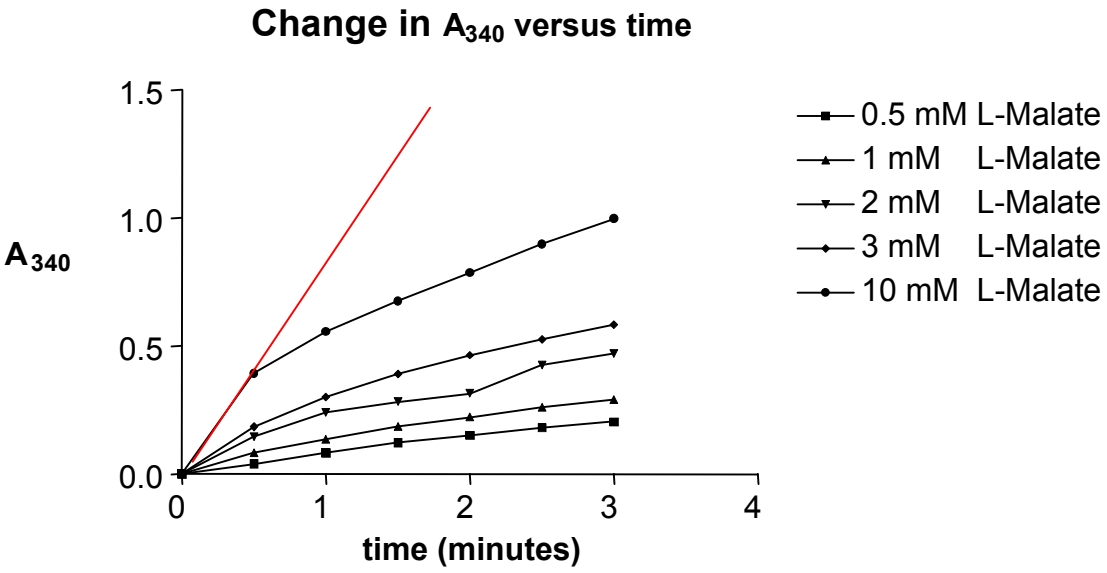
The kinetic parameters K_M and V_{max} are usually obtained from a Lineweaver-Burk plot in which the reciprocal of enzyme rate **v** is plotted against the reciprocal of substrate concentration **[S]** (hence it is often known as a double reciprocal plot). The table in the course guide is intended to guide you through the calculations. Since one of the parameters you are looking for is an intercept on the negative section of the x-axis, you should have remembered to place your y-axis in the centre of the graph paper to facilitate this.

First, the substrate concentrations. The concentrations of malate you were given constituted 1.0 ml out of a total cuvette volume of 3.0 ml. Hence with the 2.0 mM stock, the value of [S] was 2.0/3 = 0.667 mM and the reciprocal 1/[S] was 1.5 mM^{-1}.

Second the enzyme rate or reactions. Absorbance was measured at 0.5 minute intervals, enabling the change to be plotted and a line drawn and its gradient measured.

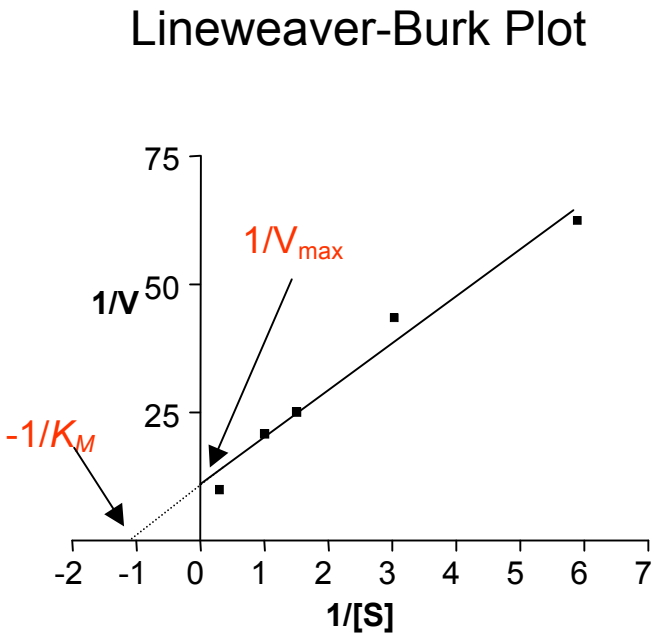
A handful of students initially took the seemingly logical step of simply taking the first and last readings and dividing the difference by 3 minutes to give the rate. However, this only works provided the two points taken are representative and the rate remains linear over the time measured. In practice occasional readings can be "off" – either because of a reading or timing error, or because something has upset it such as a bubble forming in the cuvette at that moment. By plotting a series of points such an error is easily recognised and discarded. Secondly, and certainly applicable to the reactions employing the higher concentrations of malate, the rate of reaction may decrease with time as the reaction approaches equilibrium. If this happens it is the *initial* rate which has to be measured as the Michaeis-Menton kinetics that were applied are only useful under these conditions.

An example using real data is given below:



The rate obtained is given in absorbance change per minute, and this needs to be converted to concentration units per minute. The conversion factor is the extinction constant ε (sometimes called Molar absorption coefficient) which for NADH which is 6.22 $\mu mol^{-1}.ml.cm^{-1}$. Since by the Beer-Lambert law (see practical MDL1-1, in your course guide, page 39) $A = \varepsilon \times c \times \ell$, where ℓ = 1 cm (the length of the light path through the cuvette), you can simply rearrange the equation to give: $c = A/\varepsilon$. As an example, the group above found that with 2.0 mM malate stock (0.67 mM final) the enzyme rate was 0.250 absorbance units per minute. This means a rate of change in NADH concentration (v) of 0.250/6.22 = 0.0402 $\mu mol.ml^{-1}.min^{-1}$ and a value of 1/v of 25 $\mu mol^{-1}.ml.min$.

Now the double reciprocal plot can be made:



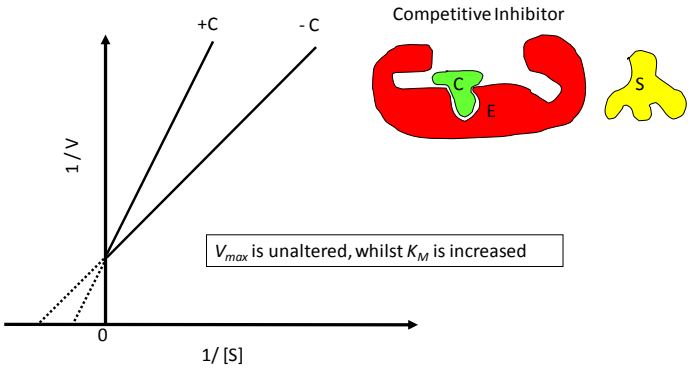
The kinetic parameters are obtained from the intercepts on the axes. The intercept on the 1/v axis is $1/v_{max}$. In this case the intercept was 11.5 $\mu mol^{-1}.ml.min$, giving a V_{max} of 0.087 $\mu mol.ml^{-1}.min^{-1}$. The intercept on the 1/[S] axis is $-1/K_M$. In this case the intercept was $-1.2\ mM^{-1}$ giving a value for K_M of 0.83 mM. Remember, the units for K_M are simply those of substrate concentration as it is

graphically defined as the substrate concentration at which ½ V_{max} is achieved. In this experiment there can be quite a lot of error in determining the intercepts, and taking the reciprocal has the effect of magnifying errors in the measured rate, especially at the lower values of [S]. Thus individual pairs may well have obtained values differing from these.

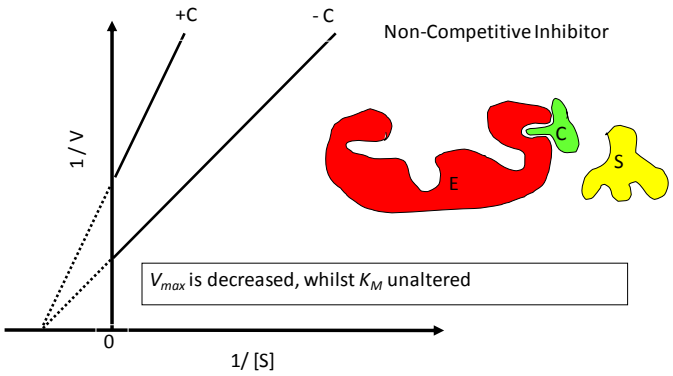
The mean values of K_M and V_{max} from a respective sample of all four are shown below, ± the standard deviation (σ) .

09/11/12 am	K_M=2.06 mM ± 1.34 and V_{max} =0.17 ± 0.07 µmol.ml^{-1}.min^{-1} (n=22)
09/11/12 pm	K_M=2.09 mM ± 1.65 and V_{max} =0.21 ± 0.16 µmol.ml^{-1}.min^{-1} (n=32)
16/11/12 am	K_M=2.90 mM ± 2.71 and V_{max} =0.16 ± 0.20 µmol.ml^{-1}.min^{-1} (n=24)
16/11/12 pm	K_M=2.31 mM ± 0.16 and V_{max} =0.11 ± 0.16 µmol.ml^{-1}.min^{-1} (n=22)

The effects of competitive and non competitive inhibitors on the parameters of K_M and V_{max} are shown below:



Effects can be overcome by increasing [S]



Effects cannot be overcome by increasing [S]

Finally the enzyme rate was also measured using D-malate instead of L-malate. The finding was that the rate was very low, typically 1-2% of that with the same (10 mM) concentration of L-malate. Hopefully you will recall from my lectures on protein structure and enzyme kinetics (Metabolism lectures 1 and 2) that enzymes are highly stereospecific!

For a more detailed look at malate dehydrogenase please check out the animation which can be found at the following URL:

https://education.med.imperial.ac.uk/Years/1-1213/MCD/animate/MD2/MalateDehydrogenase.html